

# Complement Activation Is Associated With Disease Severity in Multiple Sclerosis

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## Abstract

### Background and Objectives

Histopathologic studies have identified immunoglobulin (Ig) deposition and complement activation as contributors of CNS tissue damage in multiple sclerosis (MS). Intrathecal IgM synthesis is associated with higher MS disease activity and severity, and IgM is the strongest complement-activating immunoglobulin. In this study, we investigated whether complement components (CCs) and complement activation products (CAPs) are increased in persons with MS, especially in those with an intrathecal IgM synthesis, and whether they are associated with disease severity and progression.

### Methods

CC and CAP levels were quantified in plasma and CSF of 112 patients with clinically isolated syndrome (CIS), 127 patients with MS (90 relapsing-remitting, 14 primary progressive, and 23 secondary progressive), 31 inflammatory neurologic disease, and 44 symptomatic controls from the Basel CSF databank study. Patients with CIS/MS were followed in the Swiss MS cohort study (median 6.3 years). Levels of CC/CAP between diagnosis groups were compared; in CIS/MS, associations of CC/CAP levels with intrathecal Ig synthesis, baseline Expanded Disability Status Scale (EDSS) scores, MS Severity Score (MSSS), and neurofilament light chain (NfL) levels were investigated by linear regression, adjusted for age, sex, and albumin quotient.

### Results

CSF (but not plasma) levels of C3a, C4a, Ba, and Bb were increased in patients with CIS/MS, being most pronounced in those with an additional intrathecal IgM production. In CIS, doubling of C3a and C4a in CSF was associated with 0.31 (CI 0.06–0.56;  $p = 0.016$ ) and 0.32 (0.02–0.62;  $p = 0.041$ ) increased EDSS scores at lumbar puncture. Similarly, doubling of C3a and Ba in CIS/MS was associated with 0.61 (0.19–1.03;  $p < 0.01$ ) and 0.74 (0.18–1.31;  $p = 0.016$ ) increased future MSSS. In CIS/MS, CSF levels of C3a, C4a, Ba, and Bb were associated with increased CSF NfL levels, e.g., doubling of C3a was associated with an increase of 58% (Est. 1.58; CI 1.37–1.81;  $p < 0.0001$ ).

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## Glossary

**CAP** = complement activation products; **CC** = complement components; **CI** = confidence interval; **CIS** = clinically isolated syndrome; **DMT** = disease-modifying treatment; **EDSS** = Expanded Disability Status Scale score; **Est** = estimate; **INDC** = inflammatory neurologic disease controls; **IgG<sub>IF</sub>/IgM<sub>IF</sub>** = immunoglobulin G/M intrathecal fraction; **IQR** = interquartile range; **LLOQ** = lower limit of quantification; **LP** = lumbar puncture; **MS** = multiple sclerosis; **MSSS** = Multiple Sclerosis Severity Score; **NfL** = neurofilament light chain; **OCGB** = oligoclonal IgG bands; **PPMS** = primary progressive multiple sclerosis; **RRMS** = relapsing-remitting multiple sclerosis; **SC** = symptomatic controls; **SPMS** = secondary progressive multiple sclerosis; **ULOQ** = upper limit of quantification.

## Discussion

CNS-compartmentalized activation of the classical and alternative pathways of complement is increased in CIS/MS and associated with the presence of an intrathecal IgM production. Increased complement activation within the CSF correlates with EDSS, future MSSS, and NfL levels, supporting the concept that complement activation contributes to MS pathology and disease progression. Complement inhibition should be explored as therapeutic target to attenuate disease severity and progression in MS.

## Introduction

Complex immune mechanisms involving T and B cells, antibodies, activated macrophages, and microglia along with the vulnerability of the target tissue account for the cascade of events that leads to the development and progression of multiple sclerosis (MS).<sup>1</sup> Histologic analysis of white matter lesions in MS patients with a relatively short disease course identified 4 different patterns in early phase MS pathogenesis; complement and immunoglobulin deposition are the typical features of the predominant pattern II.<sup>2</sup> In later phases of disease, this heterogeneity of lesion morphology seems to converge into a homogenous picture with one common pattern of antibody-mediated and complement-mediated myelin phagocytosis as the dominant mechanism of demyelination.<sup>3-6</sup> These findings were corroborated by further studies, reporting similar findings of complement components (CCs) and complement activation products (CAPs) next to immunoglobulins in MS plaques and in normal-appearing white matter.<sup>6-12</sup>

A persistent intrathecal humoral immune response is a pathognomonic feature of MS: CSF-specific oligoclonal IgG bands (OCGBs) are present in up to 98%, and a quantitated intrathecal IgG and IgM synthesis according to the Reiber formula in 65%–72% and 20%–26% of patients, respectively.<sup>13,14</sup> The presence of intrathecal IgM synthesis is associated with higher disease activity and severity,<sup>13-16</sup> and other than IgG, in a dose-dependent manner.<sup>13</sup> It is further associated with disproportionately higher spinal cord involvement in early MS and increased levels of neurofilament light chain (NfL) as a measure of neuroaxonal damage, highlighting its pathogenic relevance.<sup>17</sup> As IgM is the most efficient immunoglobulin isotype for complement activation, these differences might be partially explained by an increased activation through the antibody-mediated classical pathway. Accordingly, CSF levels of C3 activation products correlate with those of IgM, but not of IgG or IgA synthesis.<sup>18</sup>

The classical complement pathway is initiated by antigen binding of IgM or IgG, and C1q then binds to the immune complex and triggers the sequential activation of subunits C1r and C1s. Activated C1s then further cascades the cleavage of components C2 and C4 into their parts, forming C4b2a, the classical pathway C3 convertase, cleaving C3 into C3a and C3b. This subsequently leads to the formation of C4b2a3b (C5 convertase). The alternative pathway serves as a powerful amplification loop. Hydrolyzed C3 binds to factor B, which is cleaved into Ba and Bb, forming the alternative pathway C3 convertase (C3bBb). All 3 complement pathways (classical, lectin, and alternative) eventually converge by assembling a C5 convertase, and C5b then triggers the formation of the terminal complement complex.<sup>6</sup>

We aimed to investigate complement activation in MS, especially in the context of an intrathecal IgM production and of those factors involved in the classical antibody-mediated pathway in patients with different disease subtypes and stages. We further investigated if there is an association with outcome parameters reflecting concurrent (Expanded Disability Status Scale score; EDSS) and future (MS Severity Score; MSSS) disease severity and NfL levels, specifically reflecting neuroaxonal damage.

## Methods

### Patients, Inclusion Criteria, and Data Collection

CSF and paired plasma samples were obtained from the CSF biobank of the University Hospital Basel from patients prospectively recruited into the Swiss MS cohort between 2012 and 2021.

We included all eligible patients with a CSF sample without erythrocyte contamination available with a first demyelinating event suggestive of MS (CIS), relapsing remitting MS

(RRMS),<sup>19</sup> secondary progressive MS (SPMS),<sup>20</sup> or primary progressive MS (PPMS)<sup>21</sup> as well as symptomatic (SC) and inflammatory neurologic disease controls (INDC).<sup>22</sup> First symptoms were defined as neurologic symptoms that lasted for  $\geq 24$  hours without fever, infection, or adverse reaction to a prescribed medication.<sup>21</sup> Patients included were not treated with corticosteroids 30 days before lumbar puncture (LP).

RRMS had to have experienced at least 2 relapses before the LP.<sup>19</sup> The inflammatory neurologic disease controls (INDC) consisted of patients with inflammatory neurologic CNS diseases different from MS.<sup>22</sup> Symptomatic controls had neurologic symptoms without pathologic objective clinical or paraclinical findings, e.g., patients with migraine.<sup>22</sup>

Demographic and clinical variables collected included sex, date of birth and in patients with CIS/MS onset of first symptoms, disease-modifying treatments, EDSS score at LP (in CIS, median 18 ([interquartile range (IQR) 9–54] days after onset), and at last follow-up visit (after median 6.3 [IQR 4.0–9.3] years). The standardized clinical assessments based on EDSS scores were performed by certified raters.<sup>23,24</sup> The MSSS was based on the EDSS at last follow-up visit.<sup>25</sup>

### Standard Protocol Approvals, Registrations, and Patient Consents

We included patients from the CSF biobank study of the Department of Neurology, University Hospital Basel, which were prospectively recruited into the Swiss MS cohort. These studies were approved by the local ethical committee, and patients were included after written informed consent.

### Samples and Routine Cerebrospinal Fluid Analysis

CSF was centrifuged (400g for 10 minutes) within 30 minutes of collection, EDTA plasma samples were collected at the same time, centrifuged (2,000g for 10 minutes), and the supernatant frozen at  $-80^{\circ}\text{C}$ . OCGBs were assessed by isoelectric focusing followed by immunofixation or immunoblotting. Testing of OCGBs was considered positive if pattern 2 or 3 (local synthesis of IgG within the CNS) was present.<sup>26</sup> CSF and serum concentrations of IgA, IgG, IgM, and albumin were measured nephelometrically. We used the derived CSF-to-serum quotients for Igs and albumin ( $Q_{\text{IgG}}$ ,  $Q_{\text{IgM}}$ ,  $Q_{\text{IgA}}$ , and  $Q_{\text{alb}}$ ) for the calculations of the intrathecal Ig synthesis according to the formulas proposed by Reiber.<sup>14</sup> The amount of intrathecal IgG, IgM, or IgA synthesis was expressed as intrathecal fraction in percentage of the total measured isotype concentration in CSF ( $\text{IgG}_{\text{IF}} = \text{IntrathecalFraction}$ ),  $\text{IgM}_{\text{IF}}$ , and  $\text{IgA}_{\text{IF}}$ ). The integrity of the blood-CSF barrier was determined by calculating the CSF/serum ratio for albumin ( $Q_{\text{alb}}$ ).<sup>26</sup>

### Measurement of Complement Components and Their Activation Products

Samples were sent to the University Hospital of Münster (Münster, Germany) on dry ice. For the quantification of complement activation products (C4a, C3a, C5a, s-C5b9, Ba,

and Bb) and components (C1q, C4, C3, C5, and Factor H and I) levels, samples were thawed at  $37^{\circ}\text{C}$  (water bath) and immediately transferred to ice to prevent complement activation and processed. Multiplex enzyme-linked immunosorbent assays (ELISAs) based on chemiluminescence were used according to the manufacturer's recommendations (Quidel, San Diego, CA; cat. number: A900, A917) to systematically profile components and activation products (in plasma and CSF samples) in duplicate determinations. Each plate contained samples from all different diagnosis groups to minimize consequences of potential interplate variation. Control samples provided by the manufacturer were included on each plate to ensure plate-to-plate consistency. Data points below lower limit of quantification (LLOQ) or above upper limit of quantification (ULOQ) were imputed (CSF: below LLOQ: 6.1%; above ULOQ: 0.6%; plasma: below LLOQ: 11.3%; above ULOQ: 0.02%; eTable 1, links.lww.com/NXI/A996). For data points above ULOQ, the respective threshold was used as a value, and for those below LLOQ, a random value between zero and LLOQ was imputed according to a uniform distribution.

### Measurement of CSF Neurofilament Light Chain Levels

CSF NfL was measured in duplicate with the NF-light<sup>®</sup> assay (Quanterix, Billerica, MA). The mean intra-assay coefficient of variation (CV) of duplicate determinations for CSF NfL was 2.5%. Few samples with intra-assay CVs  $>20\%$  were repeatedly measured. The mean interassay CVs of internal quality controls for NfL were 6.8% (8.7 pg/mL), 4.4% (17.9 pg/mL), and 6.1% (106.5 pg/mL).

### Statistics

Data are presented as median and IQR and as absolute and relative frequencies in case of categorical data. Differences in complement components and activation products' levels and the ratios C4a/C4, C3a/C3, and C5a/C5 in CSF or plasma as separate dependent variables between CIS, RRMS, SPMS, INDC vs SC (independent variables) were analyzed using linear regression models adjusted for age, sex,  $Q_{\text{alb}}$ , and treatment category at LP (vs untreated, respectively): platform (glatiramer acetate and interferons), orals (siponimod, fingolimod, dimethyl fumarate), and monoclonal antibodies (natalizumab, ocrelizumab, and rituximab) (eTable 2, links.lww.com/NXI/A996).

We analyzed associations of CSF immunoglobulin profiles with CC and CAP levels in CIS and MS combined. As intrathecal synthesis of Ig subtypes is not evenly and independently distributed and to analyze in relation to the same reference, patients were categorized in ascending order for the presence or absence of OCGB,  $\text{IgG}_{\text{IF}}$ , and  $\text{IgM}_{\text{IF}}$  ( $>0\%$  vs  $0\%$ , respectively)<sup>13,17</sup>:

1.  $\text{OCGB}^-/\text{IgG}_{\text{IF}}^-/\text{IgM}_{\text{IF}}^-$ ;  $n = 44$ ,
2.  $\text{OCGB}^+/\text{IgG}_{\text{IF}}^-/\text{IgM}_{\text{IF}}^-$ ;  $n = 50$ ,
3.  $\text{OCGB}^+/\text{IgG}_{\text{IF}}^+/\text{IgM}_{\text{IF}}^-$ ;  $n = 81$ , and
4.  $\text{OCGB}^+/\text{IgG}_{\text{IF}}^+/\text{IgM}_{\text{IF}}^+$ ;  $n = 55$

**Table 1** Demographic, Clinical, and CSF Characteristics at Lumbar Puncture

	CIS	RRMS	SPMS	PPMS	SC	INDC
<b>n</b>	112	90	23	14	44	31
<b>Demographic and clinical data</b>						
<b>Female (n, %)</b>	80 (71.4)	64 (71.1)	13 (56.5)	8 (57.1)	29 (65.9)	14 (45.2)
<b>Age at LP (y)</b>	34.4 (26.7–43.8)	38.0 (31.4–48.1)	53.7 (48.9–59.6)	48.5 (46.7–57.8)	42.7 (25.2–52.2)	57.4 (36.7–71.3)
<b>Disease duration at LP (mo)<sup>a</sup></b>	0.6 (0.3–1.8)	56.1 (15.6–91.7)	253.1 (147.0–332.1)	50.0 (15.2–81.3)	—	—
<b>EDSS at LP</b>	2.0 (1.5–2.5)	2.5 (2.0–3.5)	6.0 (4.0–7.0)	3.5 (3.0–4.0)	—	—
<b>Untreated (n, %)</b>	111 (99.1)	70 (77.8)	15 (65.2)	13 (92.9)	—	—
<b>Follow-up available (n, %)</b>	88 (78.6)	64 (71.1)	19 (82.6)	9 (64.3)	—	—
<b>Follow-up time (y)</b>	6.4 (4.0–9.1)	6.6 (3.8–9.7)	6.0 (4.3–7.1)	8.2 (5.8–10.7)	—	—
<b>CSF data</b>						
<b>Cell count</b>	4.3 (2.0–9.0)	3.0 (1.0–6.0)	1.0 (0.3–2.2)	1.7 (1.1–2.9)	1.0 (0.3–1.7)	24.6 (7.0–76.9)
<b>Q<sub>alb</sub></b>	4.5 (3.6–6.4)	5.4 (3.9–7.3)	5.5 (3.8–7.7)	5.0 (4.4–7.2)	4.3 (3.6–5.1)	9.5 (7.8–12.4)
<b>OCGB<sup>+</sup> (%)</b>	90 (80.4)	73 (82.0)	17 (85.0)	11 (78.6)	0 (0)	13 (41.9)
<b>IgG<sub>IF</sub><sup>+</sup> (%)</b>	66 (58.9)	53 (59.6)	9 (45.0)	8 (57.1)	0 (0)	7 (22.6)
<b>IgM<sub>IF</sub><sup>+</sup> (%)</b>	31 (27.7)	24 (27.0)	2 (10.0)	3 (21.4)	0 (0)	8 (25.6)
<b>IgA<sub>IF</sub><sup>+</sup> (%)</b>	5 (4.5)	7 (7.9)	1 (5.0)	1 (7.1)	0 (0)	4 (12.9)
<b>OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup> (%)<sup>b</sup></b>	22 (19.6)	16 (17.8)	3 (13.0)	3 (21.4)	44 (100.0)	17 (54.8)
<b>OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup> (%)<sup>b</sup></b>	21 (18.7)	18 (20.0)	8 (34.8)	3 (21.4)	0 (0)	5 (16.1)
<b>OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>-</sup> (%)<sup>b</sup></b>	38 (33.9)	31 (34.4)	7 (30.4)	5 (35.7)	0 (0)	1 (3.2)
<b>OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> (%)<sup>b</sup></b>	28 (25.0)	22 (24.4)	2 (8.7)	3 (21.4)	0 (0)	6 (19.4)

Abbreviations: + = presence of OCGB or IgG<sub>IF</sub>/IgM<sub>IF</sub>/IgA<sub>IF</sub>; - = absence of OCGB or IgG<sub>IF</sub>/IgM<sub>IF</sub>/IgA<sub>IF</sub>; CIS = clinically isolated syndrome; Ig G/M/A<sub>IF</sub> = immunoglobulin G/M/A intrathecal fraction; INDC = inflammatory neurologic disease controls; IQR = interquartile range; OCGB = oligoclonal IgG bands; PPMS = primary progressive MS; Q<sub>alb</sub> = albumin quotient; RRMS = relapsing-remitting MS; SC = symptomatic controls; SPMS = secondary progressive MS. Median and IQR are displayed if not mentioned otherwise.

<sup>a</sup> Since first MS symptoms.

<sup>b</sup> Five (1.6%) OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>+</sup> or OCGB<sup>-</sup>/IgM<sub>IF</sub><sup>+</sup> patients (1 RRMS, 3 SPMS, 1 INDC) and 6 (1.9%) OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>+</sup> patients (3 CIS, 2 RRMS, 1 INDC) excluded from this classification.<sup>13</sup>

Four (1.7%) patients were IgG<sub>IF</sub><sup>+</sup> or IgM<sub>IF</sub><sup>+</sup> in combination with OCGB<sup>-</sup>, and 5 (2.1%) patients had an OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>+</sup> profile and were excluded from this analysis. Using category 1 as reference, associations of the CSF Ig categories 2 to 4 (independent variables) with the 12 CC and CAP levels as well as the ratios C4a/C4, C3a/C3, and C5a/C5 in CSF or plasma as dependent variables, respectively, were analyzed by linear regression models adjusted for age, sex, Q<sub>alb</sub>, and the 3 treatment categories (vs untreated, respectively) at LP.

Associations of the 12 CC and CAP levels (separate independent variables) with EDSS at LP in CIS and with MSSS at last follow-up and CSF NfL levels in CIS/MS (dependent variables, respectively) were analyzed by using linear regression, adjusted for age, sex, and Q<sub>alb</sub>. The NfL analyses were additionally adjusted for the 3 treatment categories (vs untreated, respectively) at LP.

NfL as well as CC and CAP levels were log transformed before analysis to better comply with normal assumption. To facilitate the estimates interpretation, a logarithm with base 2 was used in those analyses including CC and CAP as separate independent variables. Estimates were further back transformed to indicate the effect in percentages. Statistical analyses were performed using R (version 3.6.3).

## Data Availability

Data are available on reasonable request.

## Results

### Patients and Clinical Data

CSF and plasma samples were available from 112 patients with CIS (2 without plasma), 90 RRMS (2 without plasma),

**Table 2** CSF Complement Components or Activation Products' Levels in CIS/MS and Inflammatory Neurologic Disease Controls (vs Symptomatic Controls)

	SC	CIS <sup>a</sup>	RRMS <sup>a</sup>	SPMS <sup>a</sup>	PPMS <sup>a</sup>	INDC <sup>a</sup>	
<b>C4a</b>	Est 95% CI		1.19 (1.02–1.40)	1.41 (1.19–1.67)	1.58 (1.22–2.04)	1.53 (1.16–2.01)	1.39 (1.10–1.75)
	<i>p</i>		0.0277	<0.0001	0.0005	0.0026	0.0054
	Con. (ng/mL)	16.5 (13.7–22.0)	20.1 (14.7–28.7)	24.4 (17.4–36.0)	30.4 (21.2–48.8)	29.9 (20.2–39.9)	31.3 (22.0–43.7)
<b>C4</b>	Est 95% CI		0.87 (0.78–0.97)	0.88 (0.78–1.00)	0.88 (0.73–1.05)	0.85 (0.70–1.04)	0.89 (0.75–1.05)
	<i>p</i>		0.0168	0.0491	0.1680	0.1076	0.1600
	Con. (µg/mL)	18.5 (15.5–20.7)	16.9 (13.6–19.7)	17.0 (13.8–19.7)	17.7 (14.4–19.2)	17.4 (14.1–18.0)	18.8 (15.6–21.5)
<b>C4a/C4</b>	Est 95% CI		1.37 (1.12–1.67)	1.59 (1.29–1.97)	1.79 (1.27–2.53)	1.79 (1.27–2.53)	1.56 (1.17–2.09)
	<i>p</i>		0.0020	<0.0001	0.0004	0.0001	0.0027
	Quotient	0.9 (0.7–1.2)	1.1 (0.9–1.9)	1.4 (1.0–2.3)	1.6 (1.3–3.4)	2.0 (1.2–2.2)	1.6 (1.0–3.1)
<b>C3a</b>	Est 95% CI		1.55 (1.29–1.86)	1.84 (1.51–2.25)	1.64 (1.22–2.22)	1.80 (1.30–2.48)	2.79 (2.13–3.66)
	<i>p</i>		<0.0001	<0.0001	0.0012	0.0004	<0.0001
	Con. (ng/mL)	1.7 (1.5–2.5)	3.1 (2.1–3.8)	3.8 (2.6–5.6)	4.0 (2.9–5.2)	4.3 (3.0–6.5)	7.6 (4.3–17.1)
<b>C3</b>	Est 95% CI		0.87 (0.65–1.16)	0.76 (0.56–1.04)	1.08 (0.68–1.72)	0.66 (0.40–1.09)	0.75 (0.49–1.14)
	<i>p</i>		0.3479	0.0896	0.7535	0.1040	0.1792
	Con. (µg/mL)	10.9 (5.5–20.3)	9.2 (4.7–20.6)	8.7 (5.2–18.4)	16.4 (9.7–28.1)	8.7 (6.3–12.6)	14.2 (6.9–21.9)
<b>C3a/C3</b>	Est 95% CI		1.78 (1.27–2.49)	2.41 (1.68–3.48)	1.52 (0.88–2.63)	2.73 (1.52–4.90)	3.73 (2.28–6.11)
	<i>p</i>		0.0009	<0.0001	0.1016	0.0009	<0.0001
	Quotient	0.2 (0.1–0.3)	0.3 (0.2–0.6)	0.4 (0.2–0.8)	0.2 (0.1–0.4)	0.5 (0.4–0.7)	0.6 (0.2–1.4)
<b>C5a</b>	Est 95% CI		1.27 (0.86–1.88)	1.04 (0.68–1.59)	1.35 (0.72–2.52)	1.04 (0.53–2.03)	1.66 (0.94–2.92)
	<i>p</i>		0.2241	0.8492	0.3528	0.9172	0.0821
	Con. (ng/mL)	0.04 (0.02–0.07)	0.05 (0.02–0.09)	0.05 (0.02–0.10)	0.09 (0.03–0.16)	0.05 (0.04–0.06)	0.15 (0.09–0.21)
<b>C5</b>	Est 95% CI		0.95 (0.81–1.11)	0.88 (0.74–1.04)	0.96 (0.75–1.24)	1.10 (0.84–1.43)	1.10 (0.88–1.38)
	<i>p</i>		0.4913	0.1349	0.7602	0.5017	0.3960
	Con. (µg/mL)	0.16 (0.13–0.21)	0.17 (0.13–0.23)	0.19 (0.14–0.28)	0.23 (0.14–0.54)	0.24 (0.20–0.36)	0.47 (0.30–0.80)
<b>C5a/C5</b>	Est 95% CI		1.34 (0.88–2.05)	1.18 (0.75–1.87)	1.40 (0.71–2.77)	0.95 (0.45–1.96)	1.50 (0.81–2.78)
	<i>p</i>		0.1697	0.4681	0.3326	0.8799	0.1959
	Quotient	0.23 (0.13–0.39)	0.23 (0.13–0.54)	0.25 (0.12–0.49)	0.23 (0.14–0.47)	0.21 (0.12–0.28)	0.32 (0.19–0.72)
<b>Ba</b>	Est 95% CI		1.16 (1.01–1.33)	1.20 (1.03–1.39)	1.18 (0.95–1.47)	1.13 (0.89–1.43)	1.90 (1.56–2.33)
	<i>p</i>		0.0377	0.0018	0.0191	0.32755	<0.0001
	Con. (ng/mL)	6.7 (5.1–8.3)	8.3 (5.8–10.3)	9.5 (6.7–12.9)	10.1 (7.3–18.2)	11.6 (7.9–13.7)	31.4 (14.7–45.1)
<b>Bb</b>	Est 95% CI		1.21 (0.96–1.53)	1.26 (0.98–1.63)	1.22 (0.83–1.79)	1.26 (0.84–1.91)	2.064(1.44–2.)
	<i>p</i>		0.1149	0.0782	0.3140	0.2654	<0.0001
	Con. (ng/mL)	3.8 (2.7–6.3)	5.1 (3.3–7.5)	6.2 (4.0–8.7)	6.6 (4.8–9.4)	6.9 (4.9–8.7)	16.0 (10.5–27.5)
<b>C1q</b>	Est 95% CI		1.06 (0.95–1.19)	1.05 (0.93–1.18)	1.10 (0.92–1.31)	1.10 (0.91–1.33)	1.57 (1.34–1.84)
	<i>p</i>		0.2673	0.4303	0.3006	0.3169	<0.0001
	Con. (µg/mL)	0.19 (0.16–0.23)	0.22 (0.17–0.25)	0.21 (0.18–0.26)	0.25 (0.19–0.31)	0.22 (0.17–0.32)	0.35 (0.27–0.51)

Continued



**Table 2** CSF Complement Components or Activation Products' Levels in CIS/MS and Inflammatory Neurologic Disease Controls (vs Symptomatic Controls) (continued)

	SC	CIS <sup>a</sup>	RRMS <sup>a</sup>	SPMS <sup>a</sup>	PPMS <sup>a</sup>	INDC <sup>a</sup>
<b>Factor H</b>						
Est 95% CI		0.93 (0.86–0.99)	0.95 (0.88–1.02)	0.89 (0.80–1.00)	0.99 (0.88–1.11)	1.15 (1.04–1.27)
<i>p</i>		0.0266	0.1474	0.0464	0.8228	0.0061
Con. (µg/mL)	2.7 (2.6–3.0)	2.7 (2.5–2.9)	2.8 (2.6–3.2)	2.9 (2.7–3.3)	3.1 (2.6–3.5)	3.8 (3.3–5.3)
<b>Factor I</b>						
Est 95% CI		0.92 (0.81–1.05)	1.00 (0.86–1.15)	0.87 (0.70–1.08)	1.04 (0.83–1.30)	1.22 (1.01–1.48)
<i>p</i>		0.2166	0.9606	0.2000	0.7541	0.0441
Con. (ng/mL)	106 (84–144)	103 (78–141)	129 (96–191)	138 (97–221)	163 (104–241)	277 (182–465)
<b>sC5b-9</b>						
Est 95% CI		1.11 (0.75–1.66)	0.88 (0.57–1.35)	1.13 (0.60–2.16)	0.95 (0.48–1.90)	1.48 (0.83–2.65)
<i>p</i>		0.6046	0.5606	0.7021	0.8888	0.1884
Con. (ng/mL)	3.0 (1.0–9.2)	3.8 (1.6–10.5)	4.2 (1.9–10.7)	5.6 (3.5–14.5)	4.7 (2.7–8.4)	18.2 (11.2–34.5)

Abbreviations: CI = 95% confidence interval; CIS = clinically isolated syndrome; Con. = concentration; Est = estimate; INDC = inflammatory neurologic disease controls; IQR = interquartile range; LP = lumbar puncture; mcg = microgram; ml = milliliter; ng = nanogram; *p* = *p*-value; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS; SC = symptomatic controls;  $Q_{Alb}$  = albumin quotient. Median and IQR raw concentrations/quotients are displayed.

<sup>a</sup> Vs symptomatic controls (SC). Estimates are adjusted for age, sex,  $Q_{Alb}$ , and treatment categories—platform, orals, and monoclonal antibodies (vs untreated, respectively). Associations of age and  $Q_{Alb}$  are displayed in eTable 4 ([links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)).

23 SPMS, 14 PPMS, 44 SC, and 31 INDC. Patients with progressive MS were older and showed higher EDSS scores than CIS/RRMS (Table 1; diagnoses of SC and INDC are listed in eTable 3, [links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)).

## Complement Components, Activation Products, and $Q_{Alb}$ , Age, and Sex

### CSF

$Q_{Alb}$  was positively associated with all complement components and activation products (all  $p < 0.001$ ) except C4, i.e., concentrations were higher with increasing  $Q_{Alb}$  (e.g., doubling of  $Q_{Alb}$  was associated with 18% higher C4a). Age increased C4a by 1% per year ( $p = 0.0065$ ); furthermore, effects of age were seen for C3, Ba, Bb, and Factor I (eTable 4, [links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)). CC and CAP were not different between sexes.

### Plasma

$Q_{Alb}$  showed no associations with plasma CC and CAP, while age was associated with Ba and s-C5b9. Male patients had 17%/13% lower C4a and C3a (both  $p < 0.01$ ) levels than female patients (eTable 5, [links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)).

## Complement Components and Activation Products' Levels in CIS/Multiple Sclerosis and Control Groups

### CSF

C4a was increased in all stages of CIS/MS vs SC, which was most pronounced in patients with progressive MS (PPMS: Est 1.53, 95% CI 1.16–2.01,  $p = 0.0026$ , i.e., levels were increased by 53% vs SC; SPMS by 58%,  $p = 0.0005$ ; RRMS by 41%;  $p < 0.0001$ ; CIS by 19%,  $p = 0.0277$ ; Table 2, Figure 1, eFigure 1 for raw values, [links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)). Instead, levels of C4 were decreased,

and subsequently, differences of the C4a/C4 ratio vs SC became more pronounced, especially in SPMS and PPMS (Table 2).

Accordingly, levels of C3a were strongly increased vs SC in CIS (55%), RRMS (84%), SPMS (64%), and PPMS (80%, all  $p \leq 0.001$ ) with even stronger effects for the C3a/C3 ratio in CIS, RRMS, and PPMS. Different from C4a, where levels in progressive MS were numerically higher than in INDC, INDC showed higher C3a levels than in all stages of CIS/MS.

Ba was increased in CIS (16%) and RRMS (20%) vs SC. No differences between CIS/MS and SC were observed for later CC and CAP C5a, C5, s-C5b9, C1q, and Factor H and I. INDC showed highest levels for Ba, Bb, C1q, and Factor H and I (Table 2).

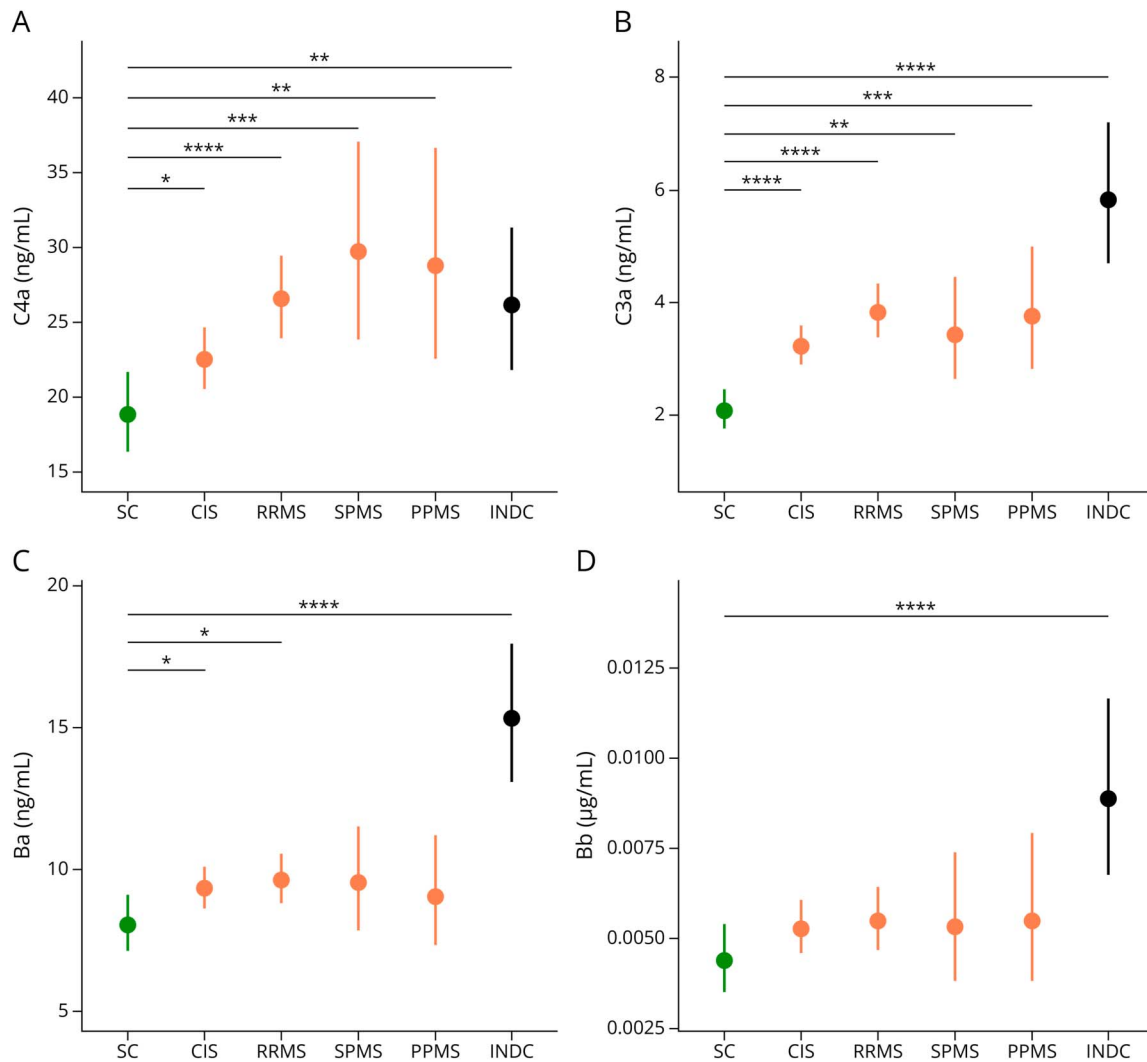
### Plasma

Few differences between groups were observed in plasma: PPMS showed 33% increased C3a ( $p = 0.0198$ ) and C3 was 14% reduced in RRMS ( $p = 0.0290$ ) in comparison with SC (eTable 5, eFigure 2 for raw values, [links.lww.com/NXI/A996](https://links.lww.com/NXI/A996)).

## CSF Complement Components and Activation Products' Levels in Ig Categories in CIS/Multiple Sclerosis

In  $OCGB^+/IgG_{IF}^-/IgM_{IF}^-$ , CSF C3a was increased by 25% ( $p = 0.0225$ ) (Table 3, Figure 2), while  $OCGB^+/IgG_{IF}^+/IgM_{IF}^-$  had higher levels for several early complement activation components vs  $OCGB^-/IgG_{IF}^-/IgM_{IF}^-$  (C4a: 28%; C3a: 67%; Ba: 20%; Bb: 37%; all  $p < 0.01$ ). These findings were consistently most pronounced in  $OCGB^+/IgG_{IF}^+/IgM_{IF}^+$  (C4a: 58%; C3a: 134%; both  $p < 0.0001$ ; Ba: 35%; Bb: 53%; both  $p < 0.01$ ). In addition, in patients with an intrathecal IgM synthesis, the C1q

**Figure 1** Adjusted Group Differences for CSF CC and CAP in Patients With CIS/MS and Inflammatory Neurologic Disease Controls vs Symptomatic Controls



Marginal effects are displayed as derived from the multivariable analyses (Table 2). (A) C4a was increased in CIS by 19% (Est. 1.19;  $p = 0.0277$ ), 41% in RRMS ( $p < 0.0001$ ), and 39% in INDC ( $p = 0.0054$ ), and this increase was most pronounced in SPMS (58%;  $p = 0.0005$ ) and PPMS (53%;  $p = 0.0026$ ) vs SC. (B) C3a levels were increased in all CIS/MS groups and INDC (CIS 55%, RRMS 84%, SPMS 64%, PPMS 80%, and INDC 179%; all  $p \leq 0.001$ ). (C) Ba was increased in CIS (16%;  $p = 0.0377$ ), RRMS (20%;  $p = 0.0178$ ), and INDC (90%;  $p < 0.0001$ ). (D) Bb levels were only higher in INDC (104%;  $p < 0.0001$ ) vs SC. CAP = complement activation products; CC = complement components; CIS = clinically isolated syndrome; Est. = estimate; INDC = inflammatory neurologic disease controls; PPMS = primary progressive MS; RRMS = relapsing-remitting MS; SPMS = secondary progressive MS; SC = symptomatic controls; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

level was increased by 23% ( $p = 0.0005$ ). OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> showed 16% decreased C4 levels ( $p = 0.0151$ ). C3a/C3 and C4a/C4 ratios supported these findings with most pronounced increases in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> (Table 3). In plasma, no such patterns were found (data not shown).

### Associations of CSF Complement Components and Activation Products With Clinical Disability Scores in CIS/Multiple Sclerosis

In multivariable analyses, average EDSS in CIS was increased by 0.32 steps per doubling of C4a (95% CI 0.02–0.62,  $p = 0.041$ ) and by 0.31 steps per each C3a concentration doubling (Est. 0.31, 95% CI 0.06–0.56,  $p = 0.016$ ). MSSS in CIS/MS at last follow-up was increased by

0.61 (CI 0.19–1.03,  $p = 0.0053$ ) steps per each doubling of C3a, and in addition, increased Ba levels were associated with a 0.74 (CI 0.18–1.31,  $p = 0.0106$ ) steps higher MSSS (Table 4, Figure 3, A and B).

### Associations of CSF Complement Components and Activation Products With NfL Levels in CIS/Multiple Sclerosis

CSF levels of several CAP were associated with higher CSF NfL levels in multivariable analysis with most pronounced effects for C4a and C3a: doubling of C3a showed 58% higher CSF NfL levels ( $p < 0.0001$ ). Similar results were seen for C4a (35%;  $p = 0.0005$ ), Ba (42%;  $p = 0.0010$ ), and Bb (17%,  $p = 0.0115$ ) (Table 4, Figure 3C).

**Table 3** Associations of CSF Immunoglobulin Categories With Complement Components and Activation Products' Levels in CSF in CIS/MS

		OCGB <sup>+</sup> /IgG <sub>IF</sub> <sup>-</sup> /IgM <sub>IF</sub> <sup>-a</sup> , n = 50	OCGB <sup>+</sup> /IgG <sub>IF</sub> <sup>+</sup> /IgM <sub>IF</sub> <sup>-a</sup> , n = 81	OCGB <sup>+</sup> /IgG <sub>IF</sub> <sup>+</sup> /IgM <sub>IF</sub> <sup>+a</sup> , n = 55
<b>C4a</b>	Est 95% CI	1.15 (0.96–1.38)	1.28 (1.09–1.52)	1.58 (1.31–1.90)
	<i>p</i>	0.1228	<b>0.0033</b>	<b>&lt;0.0001</b>
<b>C4</b>	Est 95% CI	1.09 (0.96–1.25)	1.04 (0.92–1.18)	0.84 (0.73–0.97)
	<i>p</i>	0.2000	0.5595	<b>0.0151</b>
<b>C4a/C4</b>	Est 95% CI	1.06 (0.85–1.32)	1.24 (1.01–1.52)	1.87 (1.49–2.35)
	<i>p</i>	0.6226	0.0393	<b>&lt;0.0001</b>
<b>C3a</b>	Est 95% CI	1.25 (1.03–1.50)	1.67 (1.41–1.99)	2.34 (1.91–2.82)
	<i>p</i>	<b>0.0225</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
<b>C3</b>	Est 95% CI	1.06 (0.76–1.49)	1.30 (0.95–1.77)	1.11 (0.78–1.57)
	<i>p</i>	0.7249	0.1054	0.5671
<b>C3a/C3</b>	Est 95% CI	1.17 (0.80–1.72)	1.29 (0.91–1.84)	2.11 (1.42–3.14)
	<i>p</i>	0.4190	0.1555	<b>0.0003</b>
<b>C5a</b>	Est 95% CI	0.86 (0.55–1.32)	1.06 (0.71–1.58)	1.25 (0.80–1.97)
	<i>p</i>	0.4867	0.7640	0.3246
<b>C5</b>	Est 95% CI	1.01 (0.85–1.20)	0.94 (0.80–1.10)	1.05 (0.87–1.25)
	<i>p</i>	0.9313	0.4275	0.6271
<b>C5a/C5</b>	Est 95% CI	0.85 (0.53–1.37)	1.13 (0.73–1.76)	1.20 (0.73–1.97)
	<i>p</i>	0.50623	0.5758	0.4698
<b>Ba</b>	Est 95% CI	1.08 (0.93–1.25)	1.20 (1.05–1.38)	1.35 (1.16–1.57)
	<i>p</i>	0.3298	<b>0.0092</b>	<b>0.0002</b>
<b>Bb</b>	Est 95% CI	1.21 (0.93–1.56)	1.37 (1.08–1.74)	1.53 (1.17–2.00)
	<i>p</i>	0.1577	<b>0.0096</b>	<b>0.0021</b>
<b>C1q</b>	Est 95% CI	0.99 (0.89–1.11)	1.05 (0.95–1.16)	1.23 (1.09–1.37)
	<i>p</i>	0.9235	0.3799	<b>0.0005</b>
<b>Factor H</b>	Est 95% CI	1.02 (0.95–1.09)	1.02 (0.96–1.09)	1.02 (0.95–1.09)
	<i>p</i>	0.3449	0.4855	0.6465
<b>Factor I</b>	Est 95% CI	0.99 (0.85–1.15)	1.04 (0.91–1.19)	1.11 (0.95–1.30)
	<i>p</i>	0.8625	0.5853	0.1926
<b>sC5b-9</b>	Est 95% CI	0.94 (0.59–1.51)	1.45 (0.94–2.23)	1.32 (0.81–2.15)
	<i>p</i>	0.8012	0.0943	0.2599

Abbreviations: 95% CI: 95% confidence interval; CSF IgG<sub>IF</sub>/M<sub>IF</sub><sup>+/−</sup>: presence/absence of immunoglobulin G/M intrathecal fraction; Est: estimate; OCGB<sup>+/−</sup>: presence/absence of oligoclonal IgG bands, *p*: *p*-value; Q<sub>alb</sub>: albumin quotient.

<sup>a</sup> vs category OCGB<sup>−</sup>/IgG<sub>IF</sub><sup>−</sup>/IgM<sub>IF</sub><sup>−</sup> (n = 44); adjusted for age, sex, Q<sub>alb</sub>, and treatment categories—platform, orals, and monoclonal antibodies (vs untreated, respectively).

## Discussion

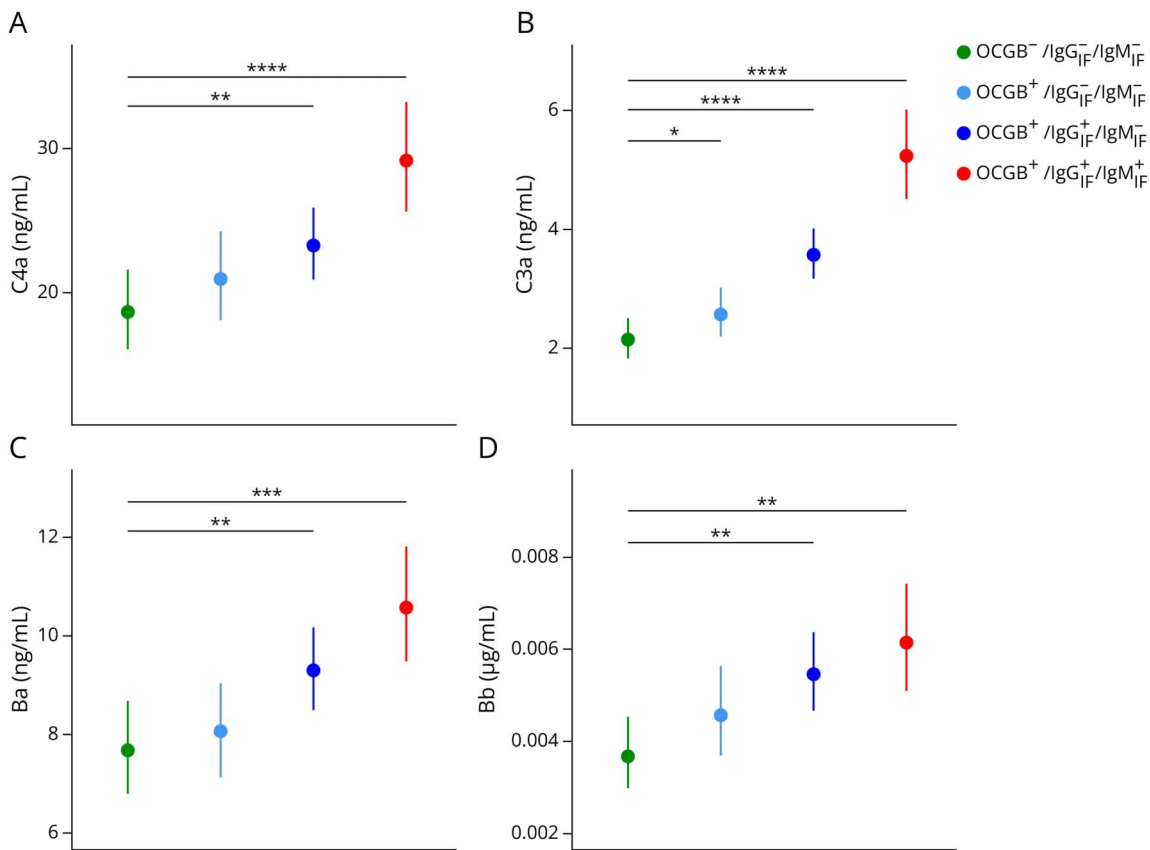
Our study demonstrates that activation products of the classical and alternative pathways, particularly C3a, C4a, Ba, and Bb, are increased in the CSF of patients with CIS and MS in comparison with symptomatic controls. Increased levels of

C4a, reflecting complement activation through the classical, antibody-mediated pathway, were most pronounced in progressive MS, even relative to INDC.

Our data corroborate earlier findings of increased levels of C3 activation products in the CSF of patients with CIS.<sup>27</sup> C4



**Figure 2** Adjusted Group Differences for CSF CC and CAP Stratified by Ig CSF Categories in Patients With CIS/MS



Marginal effects are displayed as derived from the multivariable analyses (Table 3). (A) In comparison with OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup>, C4a was increased in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>-</sup> (28% increase;  $p = 0.0033$ ) and was most pronounced in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> (58%;  $p < 0.0001$ ). (B) C3a levels were increasing along the CSF categories (vs OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup>), and this increase was most pronounced in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> (134% increase;  $p < 0.0001$ ). (C) Ba levels were increased in category OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>-</sup> (vs pattern OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup>) by 20% ( $p = 0.0092$ ) but were most pronounced in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> patients (increase 35%;  $p = 0.0002$ ). (D) Bb levels were increased in category OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>-</sup> (vs pattern OCGB<sup>-</sup>/IgG<sub>IF</sub><sup>-</sup>/IgM<sub>IF</sub><sup>-</sup>) by 37% ( $p = 0.0096$ ) but were most pronounced in OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> patients (53%;  $p = 0.0021$ ). CAP = complement activation products; CC = complement components; CIS = clinically isolated syndrome; CSF IgG<sub>IF</sub>/M<sub>IF</sub><sup>+/−</sup> = presence/absence of immunoglobulin G/M intrathecal fraction; OCGB<sup>+/−</sup> = presence/absence of oligoclonal IgG bands. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

levels were decreased, likely because of compound consumption. Later cascade complement activation products such as C5a or s-C5b9 were not increased in patients with CIS/MS, in line with previous findings.<sup>18</sup>

In accordance with others,<sup>28,29</sup> we did not find a consistent pattern of increased CC or CAP in plasma of patients with CIS/MS compared with symptomatic controls, suggesting that complement activation seems to be a part of a compartmentalized inflammatory process behind the blood-brain barrier.

We found associations between the albumin quotient, which was used as a proxy of the blood-brain barrier function and almost all CC and CAP levels in CSF. Their concentrations in plasma are in general substantially higher, so this can be explained by diffusion along the gradients. Subsequently, a proportion of CC and CAP in CSF should have their origin in the circulation.

However, it was shown that complement factors can also be produced by brain resident cells,<sup>30</sup> especially C1q and C3 seem

to play a crucial pathophysiological role and are upregulated in the more diseased MS brain.<sup>8,12,31,32</sup> The strongest increase of early complement cascade activation products (and upregulation of C1q) was observed in the OCGB<sup>+</sup>/IgG<sub>IF</sub><sup>+</sup>/IgM<sub>IF</sub><sup>+</sup> pattern, while this was less pronounced in patients with only additional IgG<sub>IF</sub> on top of OCGB, indicating main involvement of the classical pathway. The classical pathway of complement is triggered when antigen-bound IgM or IgG antibody molecules bind C1, which consists of the multimeric pattern recognition molecule C1q and a heterotetramer of the proteases C1r and C1s.<sup>33</sup> C1q binds a single IgG Fc segment with very low affinity. By contrast, antigen-driven antibody clustering and Fc-Fc interactions allow for multivalent C1q binding and optimal complement activation.<sup>34</sup> In pentameric star-shaped IgM molecules, Fc regions are already close to each other and cell surface binding of IgM readily exposes C1q binding sites.<sup>35</sup> These structural differences might account for the higher level of complement activation in patients with intrathecal IgM vs IgG production. Both antibody classes, however, require antigen binding for optimal complement activation, and the specificity of CSF-derived C1q binding of IgG and IgM remains to be elucidated.

**Table 4** Associations of CC and CAP in CSF With EDSS at LP in CIS, MSSS in CIS/MS, and CSF NfL Levels

	EDSS at LP (n = 105) <sup>a</sup>			MSSS at last follow-up (n = 168) <sup>b</sup>			CSF NfL levels (n = 235)		
	Est.	95% CI	p Value	Est.	95% CI	p Value	Est.	95% CI	p Value
<b>C4a</b>	0.32	0.02 to 0.62	<b>0.0415</b>	0.38	0.14 to 0.91	0.1558	1.35	1.14 to 1.60	<b>0.0005</b>
<b>C4</b>	-0.25	-0.60 to 0.09	0.1553	0.20	-0.44 to 0.85	0.5420	0.89	0.70 to 1.13	0.3340
<b>C3a</b>	0.31	0.06 to 0.56	<b>0.0157</b>	0.61	0.19 to 1.03	<b>0.0053</b>	1.58	1.37 to 1.81	<b>&lt;0.0001</b>
<b>C3</b>	-0.01	-0.17 to 0.16	0.9385	0.04	-0.24 to 0.31	0.7987	1.06	0.96 to 1.16	0.2500
<b>C5a</b>	-0.06	-0.19 to 0.08	0.4098	-0.04	-0.26 to 0.17	0.6879	1.04	0.97 to 1.12	0.2940
<b>C5</b>	-0.07	-0.45 to 0.31	0.7144	0.27	-0.25 to 0.79	0.3067	0.94	0.82 to 1.07	0.3369
<b>Ba</b>	0.27	-0.13 to 0.66	0.1870	0.74	0.18 to 1.31	<b>0.0106</b>	1.42	1.16 to 1.75	<b>0.0010</b>
<b>Bb</b>	0.03	-0.17 to 0.23	0.7419	0.24	-0.13 to 0.61	0.2005	1.17	1.04 to 1.32	<b>0.0115</b>
<b>C1q</b>	0.16	-0.31 to 0.63	0.4996	0.51	-0.32 to 1.33	0.2312	1.15	0.95 to 1.40	0.1613

Abbreviations: 95% CI = 95% confidence interval; CAP = complement activation products; CC = complement components; CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale score; Est = estimate; LP = lumbar puncture; MSSS = MS Severity score; NfL = neurofilament light chain; p = p-value; Q<sub>alb</sub> = albumin quotient.

For EDSS at LP (dependent variable) and MSSS (dependent variable) at last follow-up, estimates represent additive effects of doubling of each CSF CC or CAP (independent variable, besides age, sex, Q<sub>alb</sub>). For CSF NfL levels (dependent variable), estimates represent multiplicative effects of doubling of each CSF CC or CAP (independent variable, besides age, sex, Q<sub>alb</sub>, and treatment categories—platform, orals, and monoclonal antibodies (vs untreated, respectively). Separate models per CC or CAP per line.

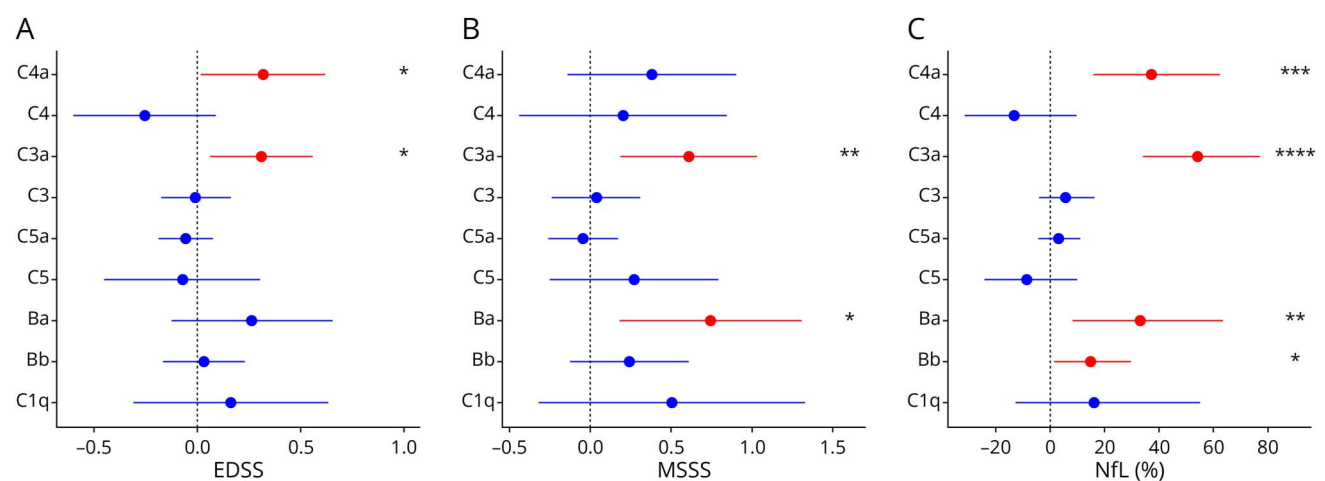
<sup>a</sup> In 7 patients with CIS, the EDSS score at LP was not available.

<sup>b</sup> Sixty-seven patients with CIS/MS had no follow-up/EDSS score at last visit, and in 4 patients, no Q<sub>alb</sub> was available.

The complement signature in patients with MS identified in this study is compatible with antibody-mediated activation of the classical pathway. In addition, we observed higher levels of activation products of the alternative pathway (AP) of complement. The AP of complement is continuously activated

and can act as an amplification loop for all 3 pathways. It is initiated by the generation and binding of C3b. Initial antibody-mediated activation of complement can, therefore, trigger alternative pathway activation and thus lead to the amplification of complement activation.<sup>33,36</sup>

**Figure 3** Adjusted Associations Between CC and CAP in CSF With (A) EDSS at LP in CIS and (B) MSSS at Last Follow-up in Patients With CIS/MS and (C) NfL Levels in CIS/MS



Estimates are displayed as derived from the multivariable analyses (A/B/C adjusted for age, sex, and Q<sub>alb</sub> and C also adjusted for treatment categories at LP) (Table 4). Separate models per CC and CAP per line are shown. (A) EDSS at LP (n = 105) was on average increased by 0.32 points per doubling of CSF C4a levels (p = 0.0415) and for C3a by 0.31 points (p = 0.0157). (B) MSSS at last follow-up (n = 168) was 0.61 points (p = 0.0053) higher when doubling CSF C3a levels and 0.74 points higher (p = 0.0106) per doubling of Ba. Marginal effects are displayed as derived from the multivariable analyses adjusted for age, sex, and Q<sub>alb</sub> (Table 4). (C) In CIS/MS (n = 235), doubling of several activation components was associated with higher CSF NfL levels (C4a: 35% higher CSF NfL levels (p = 0.0005); C3a: 58% (p < 0.0001); Ba: 42% (p = 0.0010); Bb 17% (p = 0.0115)) (Table 4). CAP = complement activation products; CC = complement components; CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale score; LP = lumbar puncture; MSSS = MS Severity Score; NfL = neurofilament light chain; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

The association of intrathecal activation products of the classical and alternative pathways (C4a, C3a, and Ba) with concurrent and future disease severity, as reflected by EDSS and MSSS, is a strong indicator for a critical role of the complement system in both inflammatory and neurodegenerative pathomechanisms of MS. It is of interest that the dose-dependent associations of NFL CSF levels with complement activation products are further indication that neuroaxonal damage may be driven by early complement cascade activation, i.e., in the absence of later cascade effector level complement activation beyond C3. These findings are paralleled by a large body of evidence from histopathologic studies suggesting complement activation as a driver of early plaque formation,<sup>2</sup> for both demyelination and axonal injury.<sup>37,38</sup> IgG and IgM colocalized with complement C3b on demyelinated axons and oligodendrocytes and antibody-antigen immunocomplexes were detected in foamy macrophages in active lesion areas.<sup>10</sup> Recombinant monoclonal IgG1 antibodies from MS patients' CSF plasmablasts bound to conformational proteolipid protein 1 membrane complexes, when injected into the mouse brain with human complement recapitulated histologic features of MS pathology including complement deposition.<sup>39</sup>

In the cortical and deep gray matter of patients with progressive MS, C1q depositions and activated complement component B and C3b were significantly increased, especially in areas with elevated numbers of complement receptor-positive microglia.<sup>12</sup> These findings are in line with a recent study demonstrating increased immunoreactivity for C1q, C4d, C3b, and Bb in thalamic lesions with an active inflammatory pathology in patients with progressive MS.<sup>40</sup>

Complement activation is not restricted to lesional tissue but extends into normal-appearing white matter, the periplaque rims, perivascular inflammatory infiltrates, and astrocytic gliosis.

Diffuse axonal injury and microglial activation as well as clusters of microglia around damaged axons coated with C3b can be found, specifically after longer disease duration.<sup>9,41,42</sup> It is however not clear whether this is causatively linked to damage or may be a reactive mechanism to remove debris of damaged axons in a chronic stage.<sup>8</sup>

One limitation of our study is the lack of MRI data, and future scientific efforts will aim at providing further validation of our study results and an investigation of treatment effects on CC and CAP levels in longitudinal CSF samples.

Preclinical studies using experimental allergic encephalomyelitis (EAE) models of MS showed that reducing complement activation can be protective from neural tissue damage. Different strategies were using a cobra venom factor as a tool to consume complement<sup>43</sup> and untargeted or targeted pharmacologic inhibition of complement activation.<sup>44</sup> Treatment with recombinant soluble human complement receptor-1 (CR1), inhibiting C3 and C5 convertase, significantly reduced clinical disease severity, inhibited CNS inflammation, almost blocked demyelination, and markedly reduced tissue deposition of C1, C3,

and C9 in an antibody-mediated demyelinating EAE rat model.<sup>45</sup> In a recent study, a C1q-blocking antibody efficiently reduced C1q staining intensity in the brain, and at a later EAE timepoint, also cells reflecting reactive gliosis in the white matter were significantly reduced. This suggested that pharmacologic inhibition of early complement activation might be a potential therapeutic avenue to address chronic inflammation in the white matter.<sup>31</sup> Hence, complement inhibition beyond the blood-brain barrier might be a novel therapeutic approach to attenuate disease severity in MS and might be one of the highly needed tools to combat ongoing smoldering progression.

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